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Papain-papaya protein interactions : resistance of papaya protein to proteolytic digestion by papain

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PAPAIN - PAPAYA PROTEIN INTERACTIONS:

RESISTANCE OF PAPAYA PROTEIN TO PROTEOLYTIC DIGESTION BY PAPAIN

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ABSTRACT

The proteolytic activity of papain was investigated with respect to its action on both natural substrates (native papaya protein and bovine serum albumin) and artificial synthetic substrate (benzoyl-l-arginine ethyl ester). Following removal of 30 percent and 40 percent of the amino acid residues from the amine terminus, the activity of the degraded papain was determined with respect to the above substrates, and compared to the activity of the intact papain molecule.

Native papaya protein showed resistance to digestion by both intact papain and 30 percent degraded papain. This resistance to digestion persisted for time periods ranging from three to six hours and was not found with the other substrates. Papaya protein did not show this resistance to digestion by 40 percent degraded papain. Digestion began immediately upon addition of 40 percent degraded papain to papaya protein. In contrast to this, papaya protein showed no resistance to digestion by other proteolytic enzymes, both of plant and animal origin.

These investigations clearly showed an interaction between native papaya protein and papain, which serves to prevent digestion of the native papaya protein by papain. This interaction was shown to be with a region of the papain between amino acid residue 62 (30 percent degraded papain) and amino acid residue 84 (40 percent degraded papain).

It is suggested that this type of interaction may be a general mechanism for the prevention of digestion of native proteins by the enzymes of the host. If this is the case, then the papain-papaya protein system could serve as an easily controlled model system for the investigation of other such enzyme systems.

ACKNOWLEDGEMENT

I would like to sincerely thank the members of my graduate committee and especially Dr. F. Sayre, for their advice and guidance throughout this investigation.

CONTENTS

INTRODUCTION

Papain has been one of the most extensively investigated proteolytic enzymes, with the possible exception of trypsin (1,2). Beginning with the studies on its isolation by Balls and Lineweaver (3,4) the final crystallization and isolation, with good yield, of papain was accomplished by Kimmel and Smith (5). This allowed the investigation of its activity by Kimmel and Smith (5), Hill and Smith (6,7,8) and Kimmel, Smith and Brown (9) in an attempt to define the enzymatic activity of papain in terms of bond specificity, physical conditions for optimum activity and kinetics. This was followed by determination of the amino acid composition and approximate location of the active site by Hill and Smith (2,8) and final elucidation of the primary structure by Husain and Lowe (10). Partial determination of the tertiary structure by X-ray studies was reported by Drenth, et. al. (11).

Although these previous studies contributed greatly to the understanding of the enzymatic activity of papain, they were all performed using synthetic amide esters, such as benzoyl-l-arginine ethyl ester (BAEE), tosyl-l-arginine methyl ester (TAME) and benzoyl-l-argininamide (BAA) (2,5,12,13). In this previous work

no attempt was made to investigate the proteolytic activity of papain with respect to the more complicated natural proteins.

E. B. Chain (14), in a symposium, discussed some future developments possible in the field of biochemistry. He stated that "once the model systems for the investigation of biological reactions are defined then we must advance to the investigation of the same reactions in terms of the more complicated complete biological systems." Cordes (15) has stated recently that:

> There has been a great deal of loose discussion concerning "Model Systems" which purport to provide insight into the mechanisms of enzymatic reactions. In this regard it is important to distinguish those cases which merely mimic enzymatic reactions in one or more respects and those which actually provide insight into enzymatic reactions.

Isolation and crystallization of papain from fresh papaya latex was first reported by Balls and Lineweaver (3,4). Their procedure required the use of fresh papaya latex and was both costly and time consuming. Modification of this procedure by Kimmel and Smith (2,5) allowed the isolation of crystalline papain, as the inactive mercury complex, from commercial dried papaya latex. Reactivation of the mercury complex was accomplished by addition of cysteine and sodium ethylenediaminetetraacetate (EDTA) (5,9). Following activation of the enzyme, enzymatic activity was assayed by following cleavage of the amide bond of benzoyl-l-

argininamide (BAA) (5). Their investigations with benzoyl-l-argininamide (BAA), carbobenzoxy-l-glutamic acid diamide (CBGADE), carbobenzoxy-l-isoglutamine (CBG), hippurylamide (HA), carbobenzoxy-l-leucinamide (CBL), carbobenzoxyglycyl-l-phenylalanine (CGP), carbobenzoxy-l-glutamyl-l-tyrosine (CGT), acetyl-l-tyrosinamide (ATA), l-leucylglycylglycine (LGG), leucylglycine (LG) and leucinamide (LA) clearly demonstrated esterase activity on substances possessing peptide or amide bonds (5). Optimum activity was found to be near pH 6 (2,5,9).

Studies by Smith, Kimmel and Brown (9) demonstrated that papain existed as a monomer, which aggregated with mercury to form a complex consisting of two molecules of papain and one of mercury. Partial activation of the mercurial complex was achieved with the addition of cysteine and full activation was obtained with cysteine and EDTA. This established the necessity for the presence of reduced sulfhydryl group(s) for full activity of the enzyme (1,2,5,9).

Studies by Bergman and Fruton (16) indicated that the bond specificity of papain was wide, splitting peptide bonds between amino acids with or without a free alpha amino group, and preferentially splitting those bonds between amino acids lacking a free carboxyl group (2,5,6). Papain also showed steriospecificity

torward the levo form of substrates (2,5,16). Again none of the studies performed for the determination of bond specificity used intact proteins as substrates (2). Hill and Smith (6,7,8) confirmed the bond specificity of papain, and Smith, et. al., (17) determined the amino acid composition.

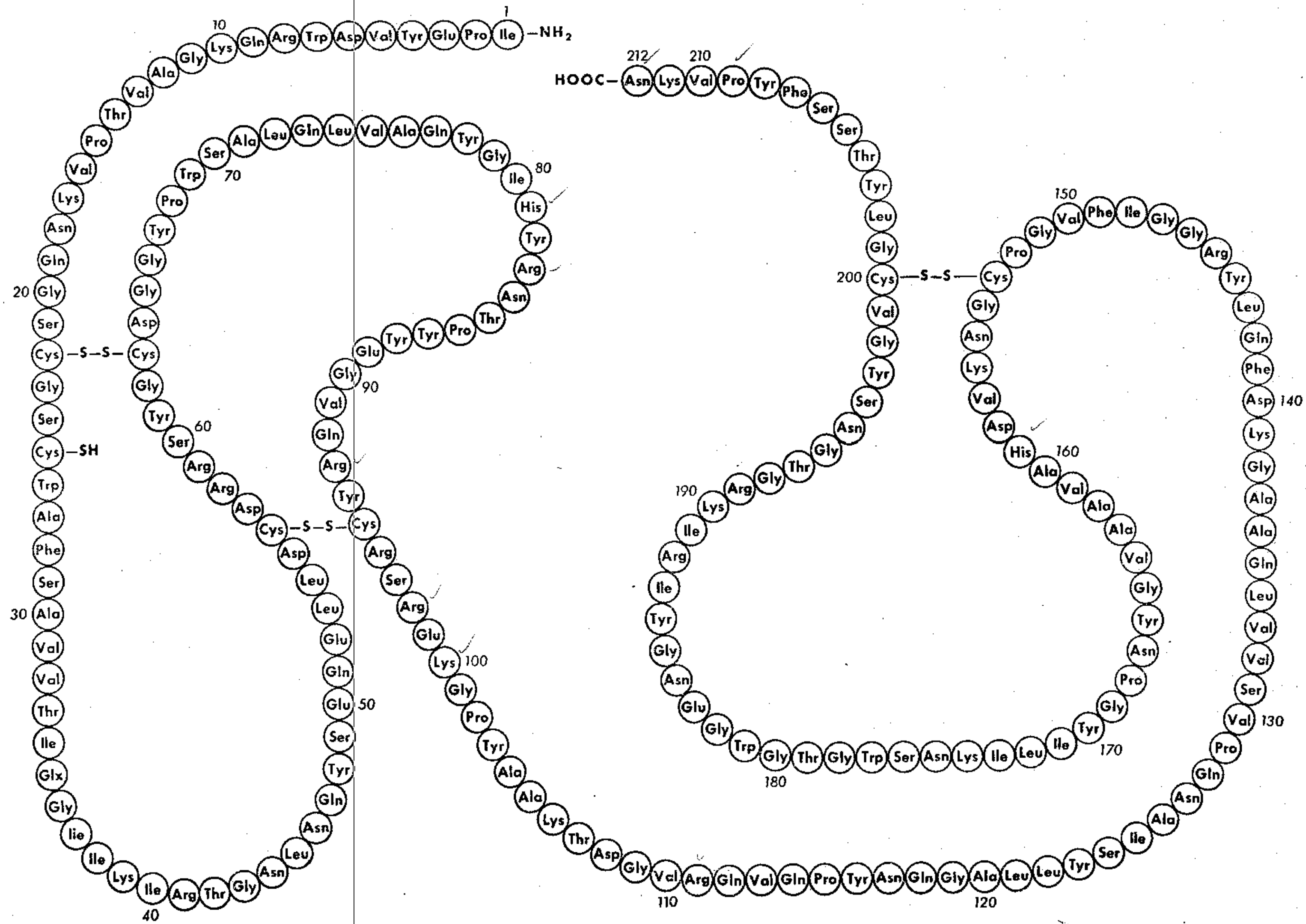
Smith, et. al., (2,17) found that one hundred amino acid residues could be removed from papain by using the proteolytic enzyme leucine amino peptidase (LAP) to remove amino acids sequentially from the amino terminus of the molecule. This modification of papain did not alter its activity torward any of the previously mentioned substrates (6,7,8,18,19). They demonstrated the presence of the intact, active papain fragment by electrophoresis, ultracentrifugation, and gel filtration chromatography following their degradation of papain. By all these methods the intact papain fragment appeared to be homogeneous (2,17). This modification of papain did not change its bond specificity, pH optimum or specific activity against the synthetic substrates used. This led them to assume that the active site of papain was near the carboxy terminus of the molecule.

The necessity of a reduced sulfhydryl group for maximum enzymatic activity was established by Smith, et. al., (17). This has been confirmed by Husain and Lowe (10). Although papain contains a

sulfhydryl group at cysteine 25, it also has three disulfide bridges which provide possibilities for the formation of sulfhydryl groups (10). An equilibrium would exist between the disulfide bridges on papain and the reduced sulfhydryl reagent in a solution containing glutathione or cysteine (2,20). This serves to convert one of the half-cystine residues in the disulfide bridge to a cysteine with a reduced sulfhydryl group.

Husain and Lowe (10) have recently completed the analysis of the primary structure of papain (Figure 1). They showed that the molecule consisted of 212 amino acid residues in a single chain. There are three disulfide bridges, between cysteine 22 and 63, cysteine 56 and 95 and cysteine 153 and 200. In addition there is a reduced sulfhydryl group at cysteine residue 25. X-ray crystal structure analysis performed by Drenth, et. al., (11) indicated a hydrophobic alpha helix region in papain running from the surface of the molecule at amino acid residue 26 to amino acid residue 41 in the interior.

Papain has been long regarded as an enzyme which is not subject to regulation by other molecules, with the exception of the reaction between the reduced sulfhydryl group(s) and mercury (2,10). The precedent has been well established for the regulation of enzymatic activity of many enzymes by the binding of ligand molecules to sites other than the active site (21,22,23).



1
Ile Pro Glu Tyr Val Asp Trp Arg Gln Lys
—NH₂
10
Gly Ala Val Thr Pro Val Lys Asn Gln Gly
20
Ser Cys Gly Ser Cys Trp Ala Phe Ser Ala
—S—S—
—SH
30
Val Val Thr Ile Glx Gly Ile Ile Lys Ile
40
Arg Thr Gly Asn Leu Asn Gln Tyr Ser Glu
50
Gln Glu Leu Leu Asp Cys Asp Arg Arg Ser
—S—S—
60
Tyr Gly Cys Asp Gly Gly Tyr Pro Trp Ser
70
Ala Leu Gln Leu Val Ala Gln Tyr Gly Ile
80
His Tyr Arg Asn Thr Pro Tyr Tyr Glu Gly
90
Val Gln Arg Tyr Cys Arg Ser Arg Glu Lys
100
Gly Pro Tyr Ala Ala Lys Thr Asp Gly Val
110
Arg Gln Val Gln Pro Tyr Asn Gln Gly Ala
120
Leu Leu Tyr Ser Ile Ala Asn Gln Pro Val
130
Ser Val Val Leu Gln Ala Ala Gly Lys Asp
140
Phe Gln Leu Tyr Arg Gly Gly Ile Phe Val
150
Gly Pro Cys Gly Asn Lys Val Asp His Ala
—S—S—
160
Val Ala Ala Val Gly Tyr Asn Pro Gly Tyr
170
Ile Leu Ile Lys Asn Ser Trp Gly Thr Gly
180
Trp Gly Glu Asn Gly Tyr Ile Arg Ile Lys
190
Arg Gly Thr Gly Asn Ser Tyr Gly Val Cys
200
Gly Leu Tyr Thr Ser Ser Phe Tyr Pro Val
210
Lys Asn
212
HOOC—

This ligand binding induces a shape change in the active site resulting in modification of the enzymatic activity. This type of regulation has been termed "allosteric" (23).

Edelman (24), Singer and Doolittle (25) and Porter and Cohen (26) have demonstrated that several types of binding sites are present on the immunoglobulins. In addition to the binding site for the antigen-antibody reaction, sites have been shown to exist which function by recognizing species differences in antigenic proteins (27). Recent work on antigenic proteins by Benjamini, et. al., (27, 28) has shown that in the case of the tobacco mosaic virus protein, the antigenic site is composed of only five amino acid residues. This site was confirmed by synthesis of the immunologically active pentapeptide (27,28). Thus the occurence of small binding and regulatory sites in protein molecules has been well established.

RESEARCH PROBLEM

It was decided to investigate the proteolytic activity of papain to determine if there did exist some regulatory or binding site other than the catalytic site, and secondly to attempt to explain the function, if any, of the one hundred amino acid residues which may be removed from the amino terminus of the enzyme molecule without significant alteration of enzymatic activity.

As all of the work to date has been performed using synthetic substrates, it was decided to investigate the activity of papain by comparing the activity with both synthetic and natural substrates. The synthetic substrate chosen was benzoyl-l-arginine ethyl ester (BAEE). The natural substrates used were bovine serum albumin and a protein mixture isolated from the papaya fruit.

It was decided to compare the activity of both intact papain and papain that had been degraded by leucine amino peptidase against these three substrates. Observed activities were then to be compared with the activity of bromelin and ficin, plant proteolytic enzymes, and trypsin, an animal proteolytic enzyme, against these same substrates.

EXPERIMENTAL

All reagents, unless otherwise noted, were prepared from reagent grade chemicals obtained from Mallinckrodit Chemical Company. Standardized acids and bases were purchased from Harleco Reagents, Inc. and the standard buffers were from Beckman Instrument Company, Inc. The enzymes were obtained from Sigma Chemical Company in the highest grade of purity available. Bovine serum albumin and benzoyl-l-arginine ethyl ester (BAEE) were also obtained from Sigma Chemical Company.

Papaya protein was isolated from locally purchased papaya fruit. Isolation of the papaya protein was carried out at 5° C. The stem of the papaya was removed along with the seeds, to eliminate the papain containing latex (19). The fruit, about 500 grams, was homogenized in 500 ml., 0.9 percent sodium chloride using a Waring Blendor. The homogenate was centrifuged until the supernatant fluid was clear. The volume of supernatant fluid was measured and sufficient ammonium sulfate added to give a solution 30 percent saturated with ammonium sulfate (29,30). The formula of Kunitz (30) was used to calculate the amount of ammonium sulfate required to give the desired saturation. This

solution was centrifuged until clear and the volume of supernatant fluid again measured. Additional ammonium sulfate was added to the supernatant fluid to give a 70 percent saturated ammonium sulfate solution. This solution was centrifuged until clear and the supernatant fluid discarded. The precipitate was dissolved in 10 ml. of 0.9 percent sodium chloride. This solution was then dialyzed overnight in an Oxford Instrument Company dialysis system against 0.9 percent sodium chloride. The solution of dissolved precipitate was then tested for the presence of ammonium ion by reaction with Nesslers Reagent (29,31). Dialysis was continued until the solution was free of ammonium ion.

The concentration of the dissolved protein precipitate was calculated from the ratio of its absorbance at 280 and 260 millimicrons, using a Beckman Model DBG spectrophotometer (32,33). The protein concentration was adjusted to 10 mg/ml of solution by dilution with 0.9 percent sodium chloride. The final protein solution was filtered using a Millipore filter, type HA, pore size 0.45 microns, to reduce the possibility of bacterial comtamination of the protein (34). The filtered protein solution was stored at 4^{o} C and used within seven days of preparation.

Determinations of enzymatic activity were

performed using a pH Stat module obtained from Radiometer Instrument Company. The module consisted of a Model TTT-11 pH meter-controller equipped with a water-jacketed glass-calomel electrode system connected to a Model ABU1 automatic burette and a Model SBR2C recorder. The water-jacketed electrode system and reaction vessel were maintained at constant temperature by a Brinkman Instrument Company Thermo-cool thermostat and cooling-heating bath. This maintained the temperature of the electrode compartment within plus or minus 0.1° C of the preset temperature. The system allowed for the maintenance of constant pH within 0.001 pH unit of the preset value by addition of acid or base in portions as small as 0.1 microliter (35,36).

Enzymatic splitting of the peptide bond was followed in the following manner. With each peptide bond split by a proteolytic enzyme there was formed, in aqueous solution, a free carboxyl group (35). At a pH higher than 3 the carboxyl group ionized giving off a hydrogen ion (37). The accompanying change in pH activated the burette of the titrator, and base was added to maintain the constant preset pH. From the normality of the base, the number of peptide bonds split may be calculated, and if the amount of base added is recorded versus time, the rate of hydrolysis may also be calculated. Thus information concerning

both the amount and rate of peptide bond splitting may be obtained from this system. Using properly standardized acid and base, it has been shown that the cleavage of the peptide bond may be followed with a sensitivity of less than 0.1 micromole of acid released (35). All determinations were performed at 25° C and the pH was maintained within 0.1 unit of the optimum pH for the enzyme being used (35).

All substrate solutions were prepared in 0.9 percent sodium chloride and contained 10 mg/ml substrate, by weight. Papaya protein solutions were used within one week of their preparation. The bovine serum albumin and benzoyl-l-arginine ethyl ester solutions were used the same day that they were prepared.

Determinations of enzymatic activity were performed in the following manner. To the reaction vessel was added 1.0 ml substrate solution and 0.1 ml of 0.5 M phosphate buffer, pH 7.4. The reaction mixture was placed in the titrator and the pH adjusted to the pH optimum of the enzyme being used by the addition of 0.1 N acid or base. Between 0.01 and 0.05 ml acid or base was usually required. The enzyme being used was then added to the reaction vessel (0.01 ml of the previously prepared 1 mg/ml solution). The titrator was then activated and constant pH maintained by the addition of 0.01 N sodium hydroxide, which had been previously standardized

versus 1.0 N hydrochloric acid. Base additions were recorded versus time.

The reaction between the enzymes and benzoyl-l-arginine ethyl ester was allowed to proceed for a sufficient time period to obtain a linear reaction rate. This allowed calculation of the specific activity of the enzyme. When bovine serum albumin and native papaya protein were used as substrates, the reaction was allowed to proceed until digestion was complete, as indicated by no furthur addition of base.

The mercurial complex of papain was degraded with leucine amino peptidase (LAP) (2,7,8,9). Leucine amino peptidase (LAP) has been shown to degrade protein molecules by removing one amino acid residue at a time from the amine terminus of the protein. The pH optimum for leucine amino peptidase (LAP) is 8.5 and this enzyme is inactive at pH 6, the optimum pH for enzymatic activity of papain (5,6,7,39,41). Since there are 212 amino acid residues in papain, and each removal of an amino acid residue releases one hydrogen ion, the amount of degradation desired may be calculated in terms of equivalents of acid released and the amount of base required for the maintenance of a constant pH. Assuming the molecular weight of papain to be 20,700, and using 10 mg mercuripapain, the digestion with leucine amino peptidase (LAP) was allowed to proceed

until the papain was 30 percent degraded (2,10). The procedure used for the degradation of papain was the same as was used for the other enzymatic digestions except that the reaction mixture was buffered to the pH optimum for leucine amino peptidase (LAP) in the same manner as described previously. When the desired amount of digestion had occured the pH was lowered to 5 with the addition of 1 N hydrochloric acid and sufficient 0.9 percent sodium chloride solution was added to give a final papain concentration of 1 mg enzyme per ml. The solution was then filtered through a Millipore filter (34). The degraded papain solution was stored at 4^{o} C and used within 24 hours of preparation. Forty percent degraded papain was prepared in a simular manner. Partially degraded papain was also isolated by gel filtration using a $\frac{1}{4}$ by 5 inch column of Sephadex G-25 (40). Ten mg of degraded enzyme was placed on the column previously equilibrated with 0.9 percent sodium chloride and eluted with 0.9 percent sodium chloride. Effluent from the column was monitored with a Turner Instrument Company Fluorometer, and the fraction containing the degraded papain was collected. The volume of the collected fraction was measured and sufficient 0.9 percent sodium chloride was added to give a papain concentration equivalent to 1 mg/ml of intact papain. The solution was filtered

through a Millipore filter, stored at 4° C and used within 24 hours of preparation. Degraded papain fragments were eluted as single peaks from the Sephadex column, indicating the homogeneity of the degraded fractions. The Sephadex gel filtration gave papain fragments free of degradation products resulting from the degradation of papain by leucine amino peptidase (LAP) (40).

The pH levels used for the enzymes investigated along with their active pH ranges is shown in Table I (19).

Table I

pH Optimum for Enzymes Used

Enzyme	pH Optimum	Active pH Range
Bromelin	6.5	5.8 - 7.0
Ficin	7.0	6.5 - 7.5
Papain	6.2	5.5 - 6.8
Papain (degraded)	6.2	5.5 - 6.8
Trypsin	7.8	7.0 - 8.5

The activity of all the enzymes listed in Table I was determined as described previously with respect to benzoyl-l-arginine ethyl ester, bovine serum albumin and papaya protein. In addition mercuripapain was assayed with the above substrates following activation of the enzyme (2). The papain fragments isolated by Sephadex gel filtration were also investigated with

respect to their activity on papaya protein.

Activation of the mercuripapain, degraded mercuripapain and isolated mercuripapain fragments was accomplished with the addition of 0.1 ml of a solution 0.1 M in glutathione and EDTA (2,20). The mercury bound to the sulfhydryl group(s) of papain was removed by the glutathione and then complexed by the EDTA, thereby leaving the sulfhydryl group(s) in the reduced form necessary for the enzymatic activity of papain and degraded papain (6,7,8,10).

Since the enzymes themselves are proteins, they may exhibit some autodigestion. The same reaction mixtures and times were used, but with the deletion of substrate to determine the extent of autodigestion of enzyme. The extent of autohydrolysis of substrate was determined by allowing the reaction mixture to run for the same legnth of time with the deletion of enzyme, but in the presence of substrate.

The amount of base uptake in the above control determinations was subtracted from the base uptake of the complete enzyme-substrate system to give the net base uptake resulting from only the enzyme-substrate interactions. Net base uptake was converted to equivalents of peptide bonds split. Milliequivalents of peptide bonds split were plotted versus time (Figures 2 - 7).

In the above manner the activity of ficin,

bromelin, trypsin, papain, mercuripapain (activated), 30 percent degraded mercuripapain (activated, 40 percent degraded mercuripapain (activated) was determined versus benzoyl-l-arginine ethyl ester, bovine serum albumin and papaya protein. The specific activity for each enzyme listed was calculated from its activity versus benzoyl-l-arginine ethyl ester (Table II, Figures 2, 3). The figures shown are typical net digestion curves for the enzymes listed.

Proteolytic activity for each enzyme versus the bovine serum albumin and the papaya protein was calculated in terms of micromoles peptide bonds split. The time required for digestion to begin was recorded (lag time). The time required for complete digestion once digestion had started was also recorded.

The two fragments of papain isolated by Sephadex gel filtration (40) were investigated with respect to their activity on papaya protein in the same manner as the other assays were performed. The time required for digestion to begin was noted, and the time required for complete digestion, once digestion began, was also noted.

Following this papaya protein was allowed to remain at reaction conditions for 6 hours and then reacted with papain, 30 percent degraded papain, 40 percent degraded papain and the isolated papain

fragments. The above enzymes were then each allowed to remain at reaction conditions for 6 hours, and then their activities determined with respect to papaya protein. The times for digestion to begin (lag time), time required for total digestion once digestion had started, and the total micromoles peptide bonds split were recorded.

RESULTS

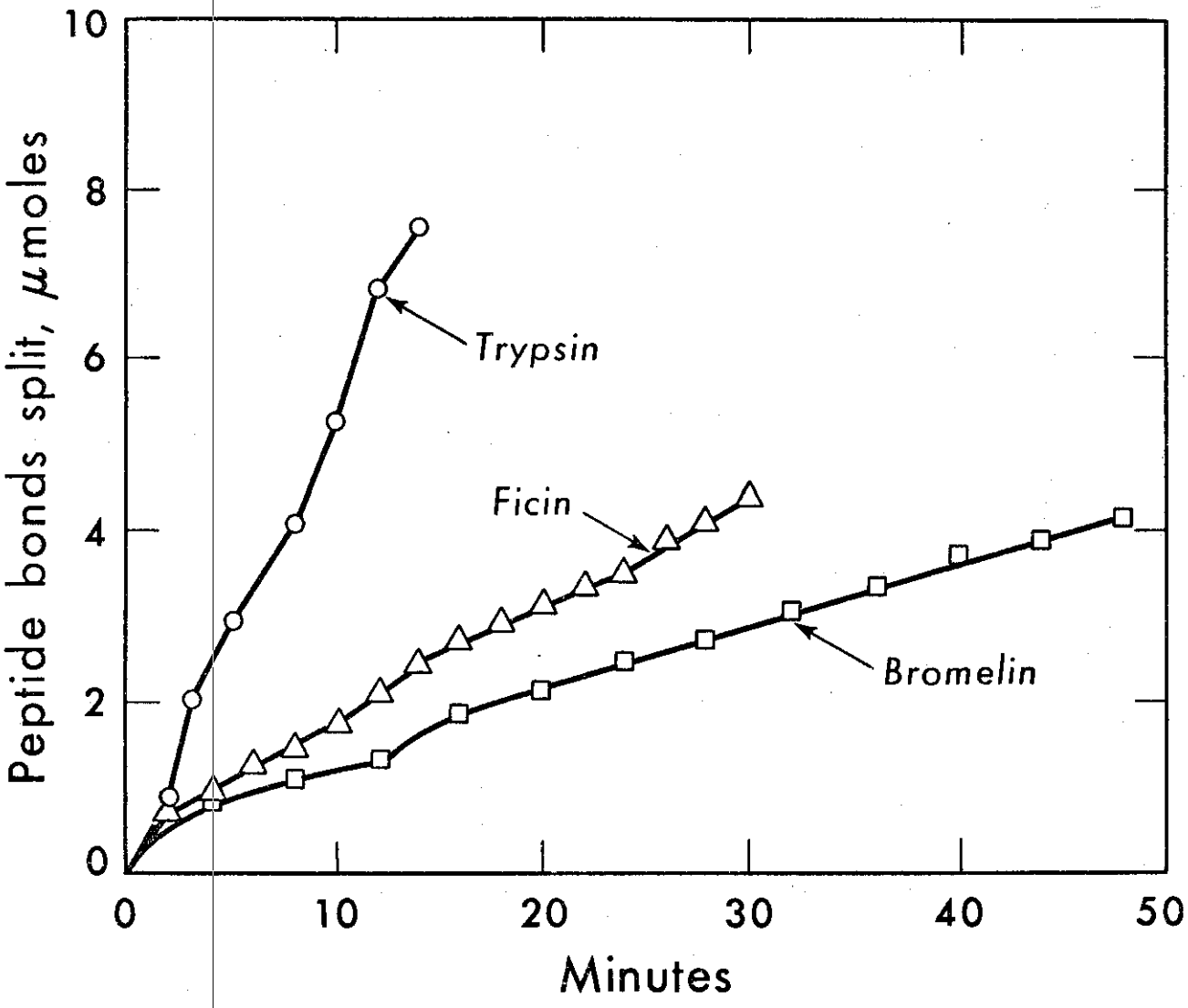
As an example of the accuracy and precision of the titrator, the base standardization prior to a typical digestion is shown in Table II.

Table II

Standardization of Sodium Hydroxide with 1.0 ml 0.01 Normal Hydrochloric Acid

Base Added	Normality
0.962 ml.	0.0104
0.952 ml.	0.0110
0.961 ml.	0.0104
0.957 ml.	0.0105
0.966 ml.	0.0103

The activity of the enzymes versus the synthetic substrate (BAEE) is shown by typical plots of proteolytic activity versus time in Figures 2 and 3. It is noted that the reaction rates are linear with proteolytic activity begining with time zero. The specific activity of the enzymes, calculated as micromoles peptide bonds split/ min/mg enzyme was calculated from five determinations on each enzyme and is shown in Table III. (page 25).


Peptide bonds split, μmoles
10
8
6
4
2
0
0
10
20
30
40
50
Minutes
Trypsin
Ficin
Bromelin

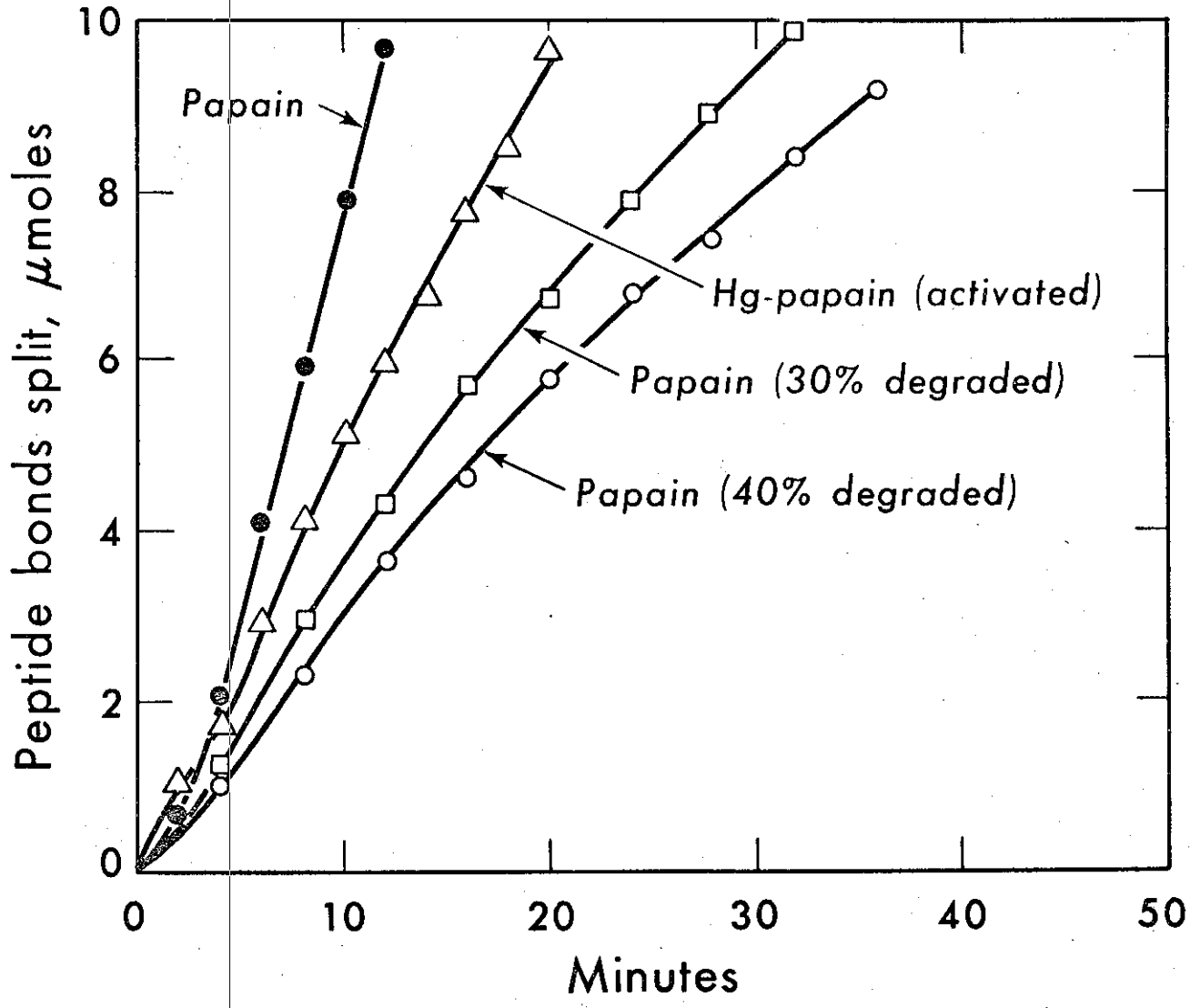

Peptide bonds split, μmoles
Minutes
Papain
Hg-papain (activated)
Papain (30% degraded)
Papain (40% degraded)
0
2
4
6
8
10
0
10
20
30
40
50

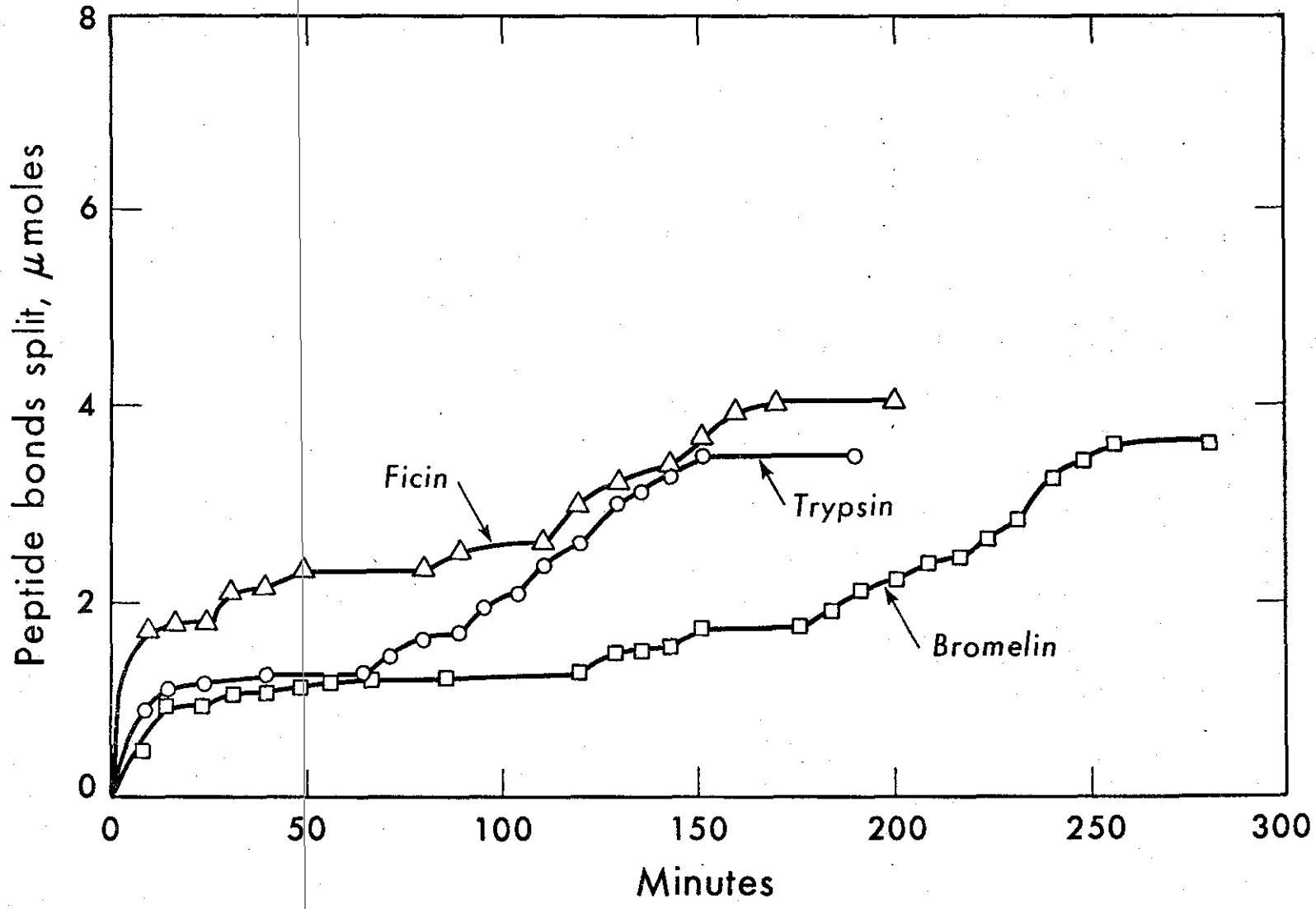
Table III

Specific Activity of Enzymes Used

Enzyme	Mean Specific Activity	Standard Deviation
Bromelin	0.9	0.10
Ficin	1.5	0.12
Hg-Papain (activated)	4.5	0.21
Papain	8.1	0.22
Papain (30% degraded)	3.1	0.20
Papain (40% degraded)	2.6	0.17
Trypsin	5.4	0.24

Typical digestion curves for the enzymes used versus bovine serum albumin are shown in Figures 4 and 5. Again it was noted that in all cases digestion began immediately. The mean time for total digestion and the mean total micromoles peptide bonds split, with the respective standard deviations are shown in Table IV (page 30). The results in Table IV represent the mean of three determinations.

The activity of the enzymes versus papaya protein is shown by representative plots of proteolytic activity versus time in Figures 6 and 7 (pages 30 - 34). It was noted that there was an extensive lag period prior to digestion of the papaya protein by papain, mercuri-papain (activated) and 30 percent degraded mercuripapain (activated). It was also noted that with bromelin, ficin, trypsin and 40 percent degraded mercuripapain


Peptide bonds split, μmoles
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2
4
6
8
0
50
100
150
200
250
300
Minutes
Ficin
Trypsin
Bromelin

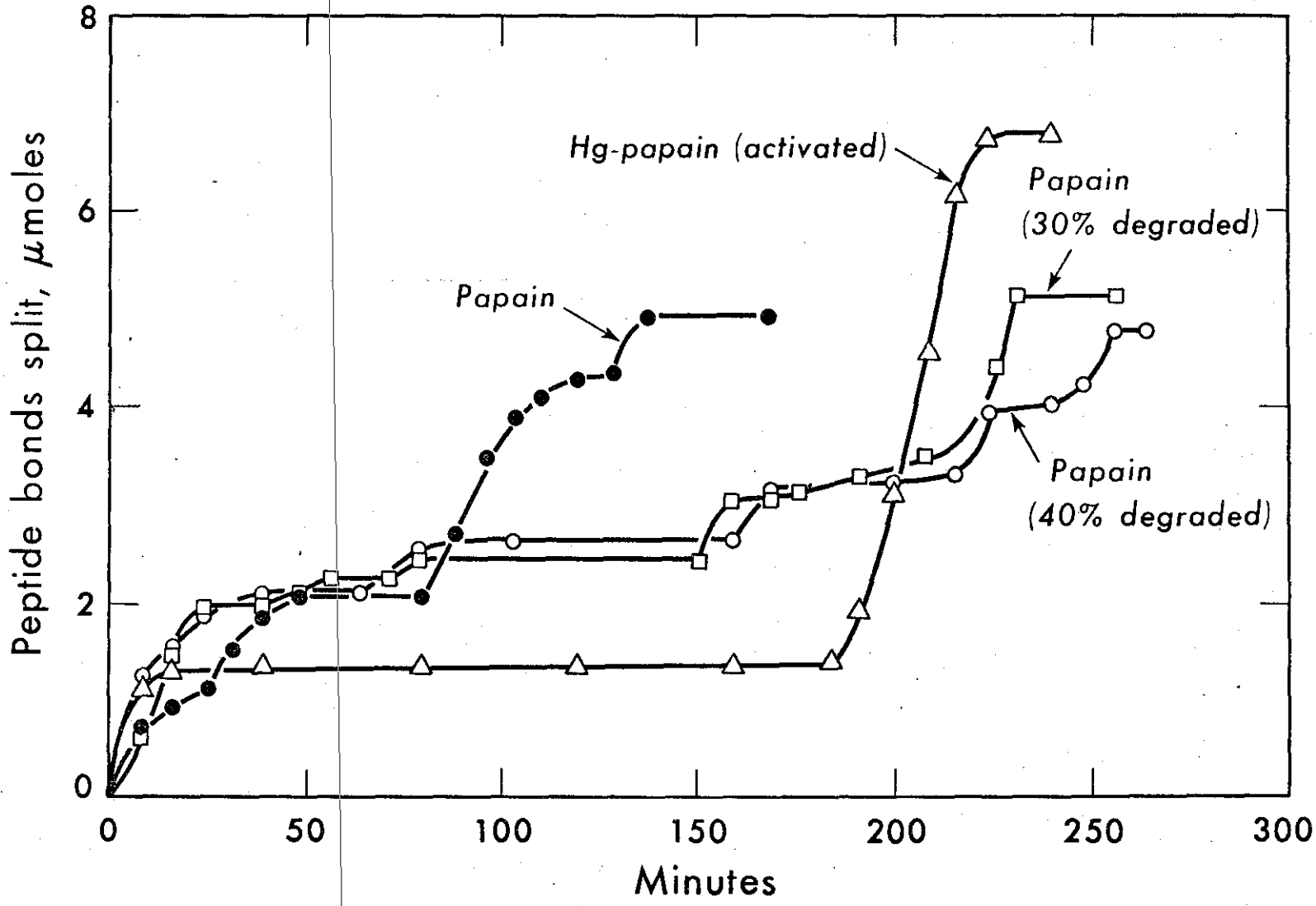
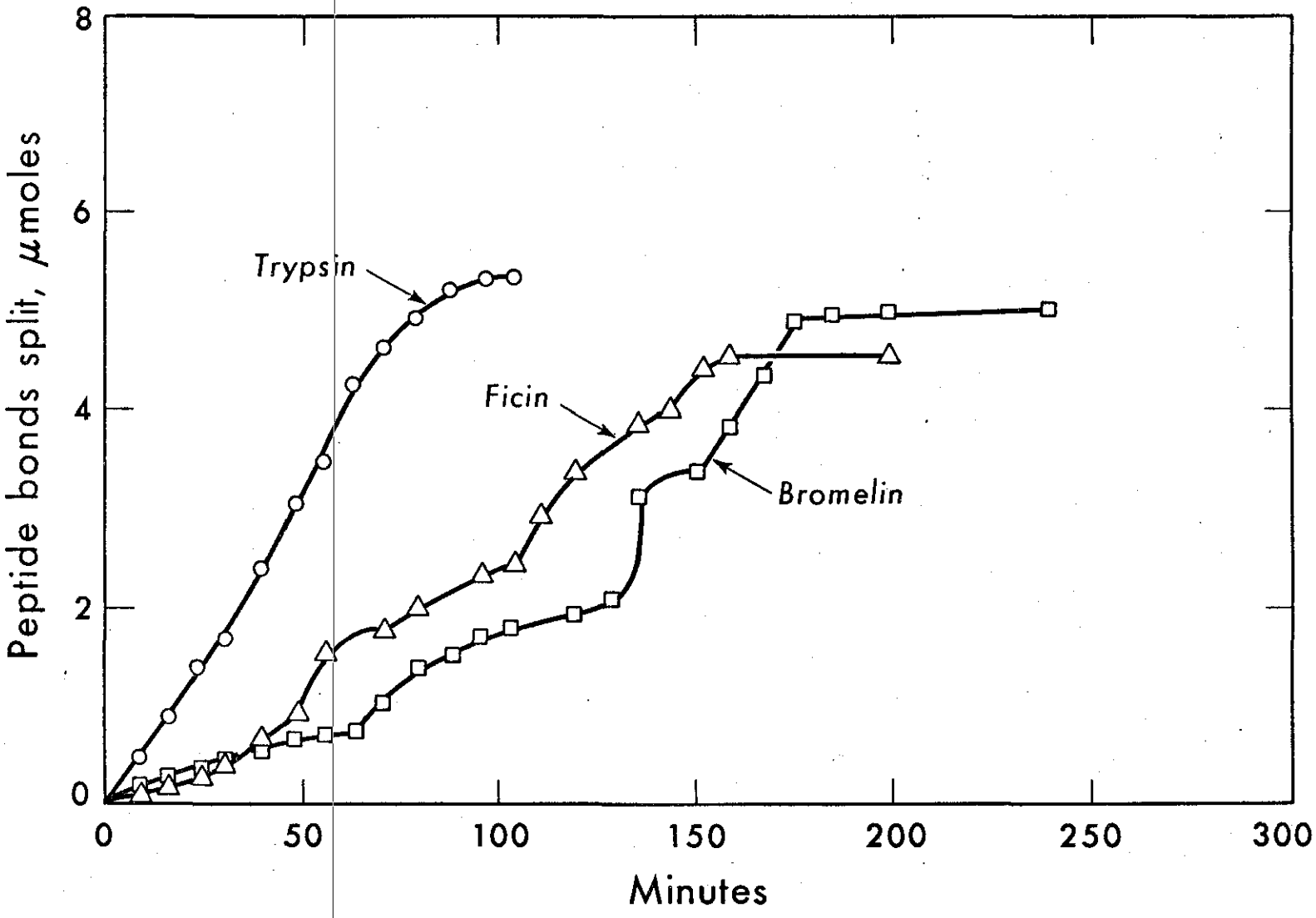
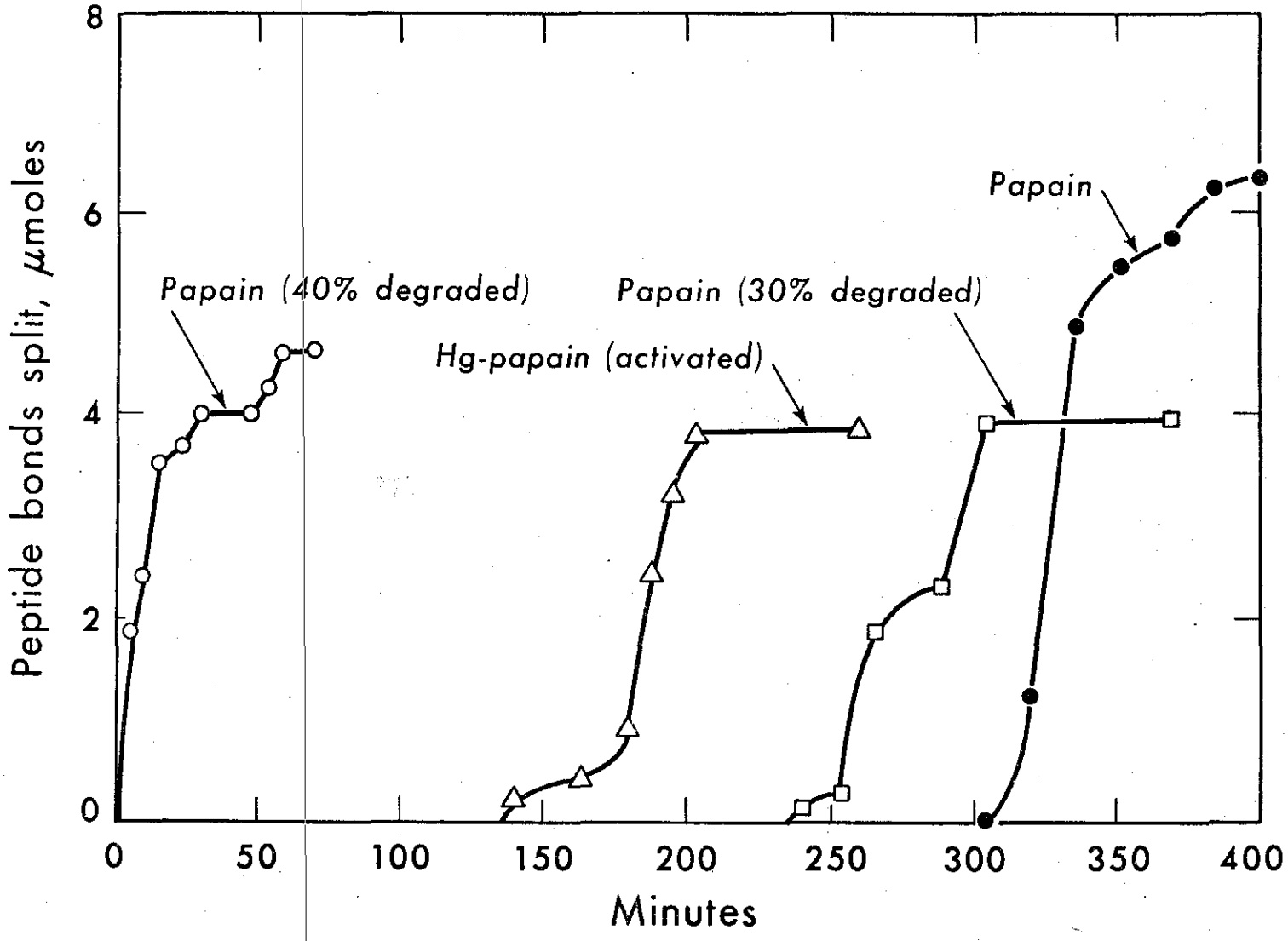

Peptide bonds split, μmoles
Minutes
0
2
4
6
8
0
50
100
150
200
250
300
Hg-papain (activated)
Papain
(30% degraded)
Papain
Papain
(40% degraded)

Table IV

Enzymatic Activity Versus Bovine Serum Albumin

Enzyme	Mean uMoles Bonds Split	Standard Deviation	Time for Total Digestion	Standard Deviation
Bromelin	3.76	0.41	255 min.	40 min.
Ficin	4.32	0.63	190 min.	36 min.
Hg-Papain (activated)	6.90	1.10	224 min.	67 min.
Papain	4.59	0.76	136 min.	29 min.
Papain (30% degraded)	5.19	0.80	260 min.	41 min.
Papain (40% degraded)	4.90	0.93	279 min.	63 min.
Trypsin	3.55	0.61	170 min.	47 min.


Peptide bonds split, μmoles
8
6
4
2
0
0
50
100
150
200
250
300
Minutes
Trypsin
Ficin
Bromelin


Peptide bonds split, μmoles
Minutes
0
2
4
6
8
0
50
100
150
200
250
300
350
400
Papain (40% degraded)
Hg-papain (activated)
Papain (30% degraded)
Papain

(activated), digestion of the papaya protein began immediately under similar reaction conditions. The lag times, total micromoles of peptide bonds split and the time required for complete digestion, after digestion began, calculated as the mean of three determinations, is shown in Table V.

It was noted that the time from start to total digestion was relatively short, except for bromelin and ficin, which had the lowest specific activities of the enzymes used. It was immediately apparent that, except in the case of 40 percent degraded mercuripapain (activated), papaya protein demonstrated resistance to digestion by papain, mercuripapain (activated) and 30 percent degraded mercuripapain (activated) for time periods ranging from 2 to 6 hours. Once digestion began however, it proceeded to completion rapidly.

Papaya protein was allowed to stand at reaction conditions for 6 hours and then papain was added. In duplicate determinations under the above conditions no lag time was demonstrated. Digestion was complete within 44 and 48 minutes; 4.01 and 4.63 micromoles of peptide bonds were split. Papain was then allowed to stand at reaction conditions for 6 hours, and then papaya protein was added. In these two determinations the lag times were 240 and 228 minutes, and then digestion proceeded to completion in 56 and 44 minutes.

Table V

Enzymatic Activity Versus Papaya Protein

Enzyme	Mean uMoles Bonds Split	Standard Deviation	Mean Lag Time	Standard Deviation	Time for Total Digestion	Standard Deviation
Bromelin	5.03	0.70	0 min.	--	217 min.	47 min.
Ficin	4.85	0.55	0 min.	--	170 min.	39 min.
Hg-Papain (activated)	3.05	0.67	195 min.	80 min.	82 min.	41 min.
Papain	6.89	0.85	310 min.	95 min.	80 min.	36 min.
Papain (30% degraded)	3.55	0.37	280 min.	63 min.	67 min.	29 min.
Papain (40% degraded)	5.07	0.55	0 min.	--	58 min.	31 min.
Trypsin	5.40	0.71	0 min.	--	96 min.	21 min.

The micromoles of peptide bonds split were 4.90 and 4.81.

Thirty percent and 40 percent degraded mercuripapain fragments, previously isolated by Sephadex gel filtration and then activated, were reacted with papaya protein. In duplicate determinations the 30 percent degraded fragment showed lag times of 204 and 304 minutes before proteolytic activity occured. The time for total digestion was 76 and 36 minutes with 5.30 and 5.02 micromoles peptide bonds split. With the 40 percent degraded fragment no lag time was demonstrated in duplicate determinations, with digestion reaching completion in 64 and 56 minutes. Micromoles of peptide bonds split were 5.10 and 5.61.

The 30 percent degraded mercuripapain fragment that had been isolated by Sephadex gel filtration and activated was then reacted with papaya protein that had been at reaction conditions for 6 hours. Under these conditions no lag time was observed in two determinations and digestion was completed in 40 and 72 minutes; 5.12 and 5.01 micromoles of peptide bonds were split respectively. When the 30 percent degraded fragment of mercuripapain that had been isolated by Sephadex gel filtration and activated was allowed to stand at reaction conditions for 6 hours there were lag times of 212 and 160 minutes before proteolytic activity was demonstrated on papaya protein.

Complete digestion required 36 and 32 minutes after digestion began. Micromoles of peptide bonds split were 5.01 and 4.57.

DISCUSSION

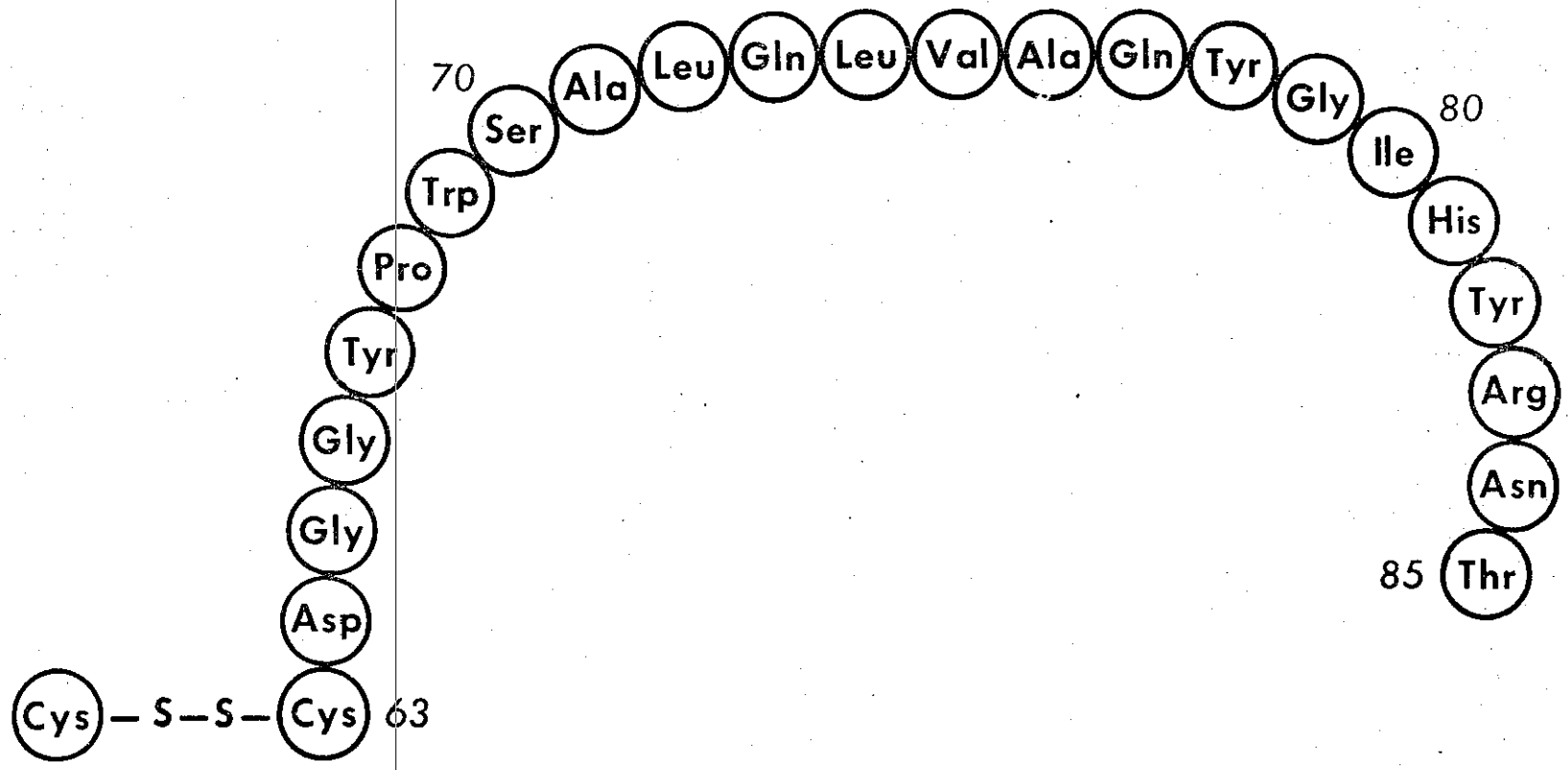
This investigation clearly showed a significant lag period before proteolytic digestion of papaya protein by papain. This lag period before proteolytic digestion was not found with any of the other substrates investigated, nor was it found when other proteolytic enzymes digested papaya protein. Removal of 30 percent of the amino acid residues from the amino terminus of the papain molecule did not eliminate or greatly change the nature of this lag period. However, the removal of 40 percent of the amino acid residues of papain, again from the amino terminus, resulted in immediate and complete digestion of the papaya protein. Removal of the products which resulted from degradation of papain did not change the nature of the observed lag period. However, experiments which allowed papaya protein to remain at reaction conditions for 6 hours, showed no lag period before digestion of the papaya protein by both intact papain and 30 percent degraded papain.

It would seem, from this investigation, that there exists somewhere between amino acid residue 62 (30 percent degraded papain) and amino acid residue

84 (40 percent degraded papain) of the papaya protein a possibility for interaction with native papaya protein that does not exist with either bovine serum albumin or the synthetic substrate, benzoyl-l-arginine ethyl ester (BAEE). This interaction prevents digestion of native papaya protein for a considerable length of time. The complementary structure in the papaya protein, which interacts with the region of the papain molecule between amino acid residues 62 (30 percent degraded papain) and 84 (40 percent degraded papain), can be altered by holding the papaya protein at reaction conditions for 6 hours, so that the lag period prior to digestion was abolished. It is well known that proteins held in a test tube environment for several hours at temperatures above refrigerator temperatures begin to undergo denaturation. It appears that digestion of the native papaya protein by papain begins only when some denaturation of the native papaya protein has occurred. The possibility that activating ions or amino acids released during the degradation of papain by leucine amino peptidase could be responsible for eliminating the lag period was eliminated by the studies with papain fragments isolated by Sephadex gel filtration. The investigations with bovine serum albumin indicated that this interaction was more than a simple masking of the active site of papain by a large molecule. If it were

only a simple masking due to size of the substrate molecule, then another large protein molecule such as bovine serum albumin should also mask the active site(s) on papain and the other enzymes investigated. If the delay in digestion of papaya protein by papain was due to a masking of the active site(s) of the papain by the papaya protein then it is indeed unique to papain and papaya protein. If this were not so, there is the possibility that papaya protein could also have masked the active site(s) on the other enzymes investigated, and this was clearly not the case. The other enzymes, in sharp contrast to intact papain, gave immediate and rapid digestion of the native papaya protein.

Figure 8 shows the amino acid sequence within this region of the papain molecule. There is a sulfhydryl group at cysteine 63 which forms a disulfide bridge with cysteine 22. Upon the removal of 30 percent of the amino acid residues, and following activation of the degraded enzyme, the sulfur of cysteine 63 becomes a reduced sulfhydryl group. The proline residue at position 68 would cause a disruption of the alpha helix portion of the molecule in this region. Following this, there is a hydrophobic area composed of 12 amino acid residues through residue 80. After this hydrophobic area is a histidine residue with a ionizable "R" group,


Cys
S—S
Cys
63
Asp
Gly
Gly
Tyr
Pro
Trp
Ser
70
Ala
Leu
Gln
Leu
Val
Ala
Gln
Tyr
Gly
Ile
80
His
Tyr
Arg
Asn
85
Thr

having a pK in the region of pH 6, very near the pH for optimal activity of papain (35). At position 83 is an arginine residue having a guanidino "R" group which is protonated at pH 6 (37).

The existance of modifying sites separate and distant from the site(s) of catalytic activity on enzyme molecules has been well established (21,25). It has been shown that these sites function by binding ligand (modifier molecules) which, upon binding, cause a shape change at the active site(s) thereby either diminishing or enhancing the binding of substrate (21,23).

Work with immunoglobulins has shown that there are reactive sites whose function is species recognition of the antigenically active proteins and which are separate from the site of antigen-antibody binding (24,25). These recognition sites on the immunoglobulins infer complementary sites or regions on the antigenic protein which will bind to the species recognition site. One such site has been identified in the case of the tobacco mosaic virus coat protein (27,28). This site has been isolated and its amino acid sequence determined (27,28). This sequence of five amino acids was synthesized and demonstrated the same antigenic activity as the intact tobacco mosaic virus protein. This region of the tobacco mosaic virus protein has

been postulated as responsible for the stimulation of antibody production in the host organism (27,28). It has also been demonstrated that this region is a pentapeptide and has a precisely fixed amino acid sequence (28). This sequence is so precise that substitution of a single amino acid, or reversal in sequence of any two amino acids, results in the elimination of its antigenic activity (28).

These studies indicated that the region of papain between amino acid residue 62 (30 percent degraded papain) and 84 (40 percent degraded papain) had a complementary site on native papaya protein. These complementary sites on papain and papaya protein may interact with each other in a manner analogous to the interaction of the tobacco mosaic virus antigenic site with its host protein.

It was determined that there is an interaction unique to papain and native papaya protein, which serves to delay the proteolytic activity of papain on native papaya protein for a significant length of time. If this indeed is a protective mechanism to prevent the digestion of papaya protein by its own enzyme, it could occur through several means. The interactions between the region of the papain molecule (amino acid residues 62 - 85) and the papaya protein could force the active site of papain into a nonactive spatial configuration (21,22,23). This

would prevent the precise spatial fit between the active site on the enzyme and the substrate necessary for enzymatic activity. It could also alter rigidity in the papain molecule, and thus prevent the so called "induced fit" from taking place, as proposed by Koshland (42) as a mechanism for enzyme-substrate binding. An induced fit mechanism has been recently demonstrated with lysozyme by Chipman and Sharon (43). Using X-ray crystallographic studies they demonstrated that binding of substrate at a single point in the active center of lysozyme changed the shape of the active site. This shape change in the active center facilitated additional binding between substrate and enzyme (43).

If these studies demonstrated a mechanism for protection of native proteins from digestion by their host's enzymes then they could serve as a model for the investigation of other enzymatic systems.

There are many examples of enzymes in easy contact with their host proteins, and yet no digestion of the protein normally occurs. Digestion can occur under circumstances which give rise to denaturation of the host proteins, or chemical modifications of the enzymes. Two striking examples are acute pancreatitis and peptic ulcer. In acute pancreatitis the protein of the pancreas is digested by the trypsin produced in the pancreas (44,45). In this example it is a

change in the enzyme that allows the digestion of the host proteins. In the case of peptic ulcer the analogy is even more striking. In the normal stomach, under the most extreme conditions of pH, there is no apparent digestion of stomach by pepsin, even though the enzyme demonstrates extensive proteolytic activity with respect to ingested protein. The exception is the case of peptic ulcer, where rapid and extensive digestion of the stomach by the host's pepsin occurs, but preceeded first by conditions which serve to enhance denaturation of the protein in the stomach lining (46,47).

In any case, this investigation served to indicate the necessity for the investigation of enzymatic systems in the context of their relationship to their entire host environment, (in as far as possible). Isolated or synthetic systems can serve to give an understanding of mechanisms of enzyme action. The understanding of greatest significance, however, may well lie in the interactions between specific enzymes and the entire host system. Care must be taken to investigate enzymatic reactions in their physiological context as well as in isolated systems (14,15). It is felt that the papain-papaya protein system may provide an easily controlled experimental model system for such investigations.

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